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# In vivo cellular uptake of bismuth ions from shotgun pellets

**M. Stoltenberg, L. Locht, A. Larsen, D. Jensen and G. Danscher**

Department of Neurobiology, Institute of Anatomy, University of Aarhus, Denmark

**Summary.** Shotgun pellets containing bismuth (Bi) are widely used and may cause a rather intense exposure of some wild animals to Bi. A Bi shotgun pellet was implanted intramuscularly in the triceps surae muscle of 18 adult male Wistar rats. Another group of 9 animals had a Bi shotgun pellet implanted intracranially in the neocortex. Eight weeks to 12 months later the release of Bi ions was analysed by autometallography (AMG) of tissue sections from different organs (brain, spinal cord, kidney, liver, testes). In the group with intramuscular Bi shotgun pellets no AMG staining could be found for the first 2-4 months; 6 months after exposure Bi was traced in the kidney. Twelve months after the implantation the kidneys were heavily loaded and Bi was also traced in testosterone-producing Leydig cells, in glial cells and in neurons of brain and spinal cord. In the central nervous system (CNS) motor neurons were the most loaded. In rats with intracranially implanted shotgun pellets a massive uptake of Bi was observed involving both glia and neurons throughout the brain. The cells close to the shotgun pellet had the highest uptake. The animals showed a pronounced Bi uptake in the ependyma cells lining the ventricular system and in the cubic epithelia covering the choroid plexus. Dissemination of Bi ions to the rest of the body was demonstrated by AMG tracing of Bi accumulations in the tubular cells of the kidney. These findings emphasize that metallic Bi, including shotgun pellets, represents sites of intense Bi pollution if implanted or shot into a living organism, and further that such metallic Bi bodies, if they enter the CNS, cause a spread of Bi ions throughout it.

**Key words:** Autometallography, Animal Model, Rat, Toxicity, Heavy Metal

*Offprint requests to:* Meredin Stoltenberg, MD, PhD, Department of Neurobiology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark. Fax: (+45) 8942 3060. e-mail: ms@neuro.au.dk

## Introduction

The popularity of "recreational" hunting involves the fact that a high number of wild animals, and in particular birds, are wounded by shotgun pellets (Noer et al., 1996; Sanderson et al., 1998), but survive for shorter or longer periods of time. Official reports state that up to 37% of the game animals in Denmark have been wounded by shotgun pellets (Noer et al., 1996). Because shotgun pellets of lead are forbidden in many countries, including Denmark, shotgun pellets made of other metals have gained momentum. Bismuth shotgun pellets have become quite popular, also because of their excellent ballistic qualities (unofficially the annual Danish use is estimated to be 30-40 tons) and their "softness" (i.e., they cause no damage to wood and thus sawmills). This implies a rather intense exposure of the hunted game and other organisms of the hunting grounds.

Until recently, only quantitative techniques were available for Bi measurements. However, the introduction of autometallography (AMG) as a histochemical tool for Bi tracing in tissue sections at both light microscopical and ultrastructural levels has changed the situation completely (Ross et al., 1994; Danscher et al., 2000). Bismuth clusters composed of bismuth-sulphide molecules are silver-enhanced when placed in an AMG developer. By analysing isolated AMG silver grains with the multielement technique "proton-induced X-ray emission" (PIXE), we have proved that such grains are caused by bismuth-sulphide clusters (Danscher et al., 2000). Bismuth shotgun pellets contain a minor amount of tin. However, this metal cannot be AMG silver-enhanced (Pamphlet et al., 2000).

Bismuth is still believed to have a low toxicity or even to be inert to the environment, as no serious signs of intoxication or unpleasant side effects have been reported, apart from the French outbreak of Bi brain-disease (Martin-Bouyer, 1976; Martin-Bouyer et al., 1981). The present study, which demonstrates Bi ion release from Bi shotgun pellets in a living animal, and the recent findings of Bi-caused changes in sex hormone levels (Stoltenberg et al., 2002) and neuron death

(Stoltenberg et al., 2003) may, however, increase the interest and research as to the nature of this widely used metal.

## Materials and methods

Forty-two male Wistar rats (Møllegaard Breeding Center Ltd., Ejby, Denmark), 3 months old, were used in the experiment. The rats were kept in a room with a 12 h light/dark cycle at a temperature of 21-22 °C and had a continuous supply of Altromin no. 1324 rat and mouse diet and tap water. The study was undertaken in accordance with the Danish and University of Aarhus guidelines for animal welfare.

### *Intramuscular emplacement of 1 shotgun pellet*

Eighteen rats were randomly divided into 6 groups of 3 animals; twelve rats were randomly divided into 6 groups of two animals and served as corresponding controls. The rats were anaesthetized with halothane. Temgesic® (buprenorphin) was used as an analgeticum. The left hind limb was shaved and an incision was made to expose the triceps surae muscle. One ethanol-cleaned pellet was placed in the muscle, and the skin wound was closed. Incisions were closed with two Clay Adams stainless steel autoclips (Beckton Dickinson), which were removed 7 days later. The control rats were sham operated.

The six groups of exposed animals were killed with an interval of two months, resulting in exposure periods of 2, 4, 6, 8, 10, and 12 months respectively. The controls were killed at similar intervals.

### *Intracranial emplacement of 1 shotgun pellet*

The rats were anaesthetized with 50 mg/kg Mebumal before being placed in the stereotaxic instrument (C.H. Stoelting Co, cat.no.51400). Temgesic® (buprenorphin) was used as an analgeticum. An incision in the skin was made to expose the scalp. A hole was made through the scalp with a drill (coordinates: A 8.6, V 5.2, L 3.4), and the ethanol-cleaned pellet was placed in the cerebrum with a pair of tweezers (Fig. 2). Incisions were closed with two Clay Adams stainless steel autoclips (Beckton Dickinson), which were removed 7 days later. The whole procedure was made under sterile conditions.

Twelve rats were divided into four groups of 3 animals, and each rat had one Bi shotgun pellet implanted in the neocortex. Group 1 survived for 1 month; group 2 for 2 months; and group 3 for 21/2 months (3-month survival was planned originally, but the rats started to be aggressive, shiver and evolve cramps; it was therefore decided to kill them). Group 4 were sham operated and served as controls.

### *Tissue handling*

Before they were killed the rats were anaesthetized with Mebumal and perfused through the left ventricle of the heart with 500 ml of 3% glutaraldehyde in phosphate buffer. The liver, kidneys, testes, brain, and l spinal cord were removed and post-fixed in glutaraldehyde for 48 hours.

The AMG developer consisted of 60 ml gum arabic solution and 10 ml sodium citrate buffer (25.5 g of citric acid•1$H_2O$ + 23.5 g sodium citrate•2 $H_2O$ to 100 ml distilled water). Immediately before use 15 ml reductor (0.85 g of hydroquinone dissolved in 15 ml distilled water at 40 °C) and 15 ml of a solution containing silver ions (0.12 g silver lactate in 15 ml distilled water at 40 °C) were added and the AMG developer was thoroughly stirred (Danscher et al., 2000).

The glass slides were put in a jar filled with the AMG developer and placed in a water bath at 26 °C. The entire set-up was covered with a dark hood. During the AMG development an electric device shook the jars gently. After 60 minutes the AMG development was stopped by rinsing the slices in water and afterwards replacing the developer with a 5% sodium thiosulphate solution for 10 min, the AMG stop bath solution. The jars were then placed under gently running ion-exchange water for 20 minutes. The sections were counterstained with a 0.1% aqueous toluidine blue solution (pH 4.0), dehydrated in alcohol to xylene, and ultimately embedded in DEPEX and covered with a coverglass. Black silver grains represent silver-encapsulated bismuth-sulphide molecules. All procedures and protocols have previously been described in detail (Danscher et al., 2000).

### *The Bi shotgun pellets used*

The average weight of 10 shotgun pellets (Eley 12) was 0.17 g (SD 0.01 g). Proton-induced X-ray emission (PIXE) has previously demonstrated that the pellets contained 91% Bi and 9% tin (Pamphlett et al., 2000).

## Results

### *General observations*

Three of the rats with an intracranial shotgun pellet implantation exhibited aggressive behaviour after 9 weeks and started to shiver; 2.5 months after the implantation they developed cramps and were therefore sacrificed. The rest of the animals showed no ill-effects from the operation and gained weight, moved, ate and drank like the control animals.

No AMG grains were seen in the control tissue sections from any of the sham-operated mice.

### *Bismuth accumulation in the animals intramuscularly exposed to Bi shotgun pellets*

Rats that were sacrificed before 12 months had no $Bi^{AMG}$ staining in the CNS, whereas animals exposed for 12 months revealed Bi in the motor neurons of the spinal

cord (Fig. 1A) and brain stem, in particular in the facial nucleus (Fig. 1B). The CNS was otherwise unstained apart from a sporadic Bi uptake of glial cells throughout the CNS.

In the kidneys, Bi uptake appeared to be time-dependent. The animals exposed to Bi for 6 months showed a sporadic staining of $Bi^{AMG}$ in the tubular cells, 8 months of exposure caused a rather intense accumulation of AMG grains, and after 1 year the tubular epithelial cells were heavily loaded with Bi (Fig. 1C). The glomeruli were always void of AMG staining.

One year after Bi shotgun implantations, Bi could be traced in the cytoplasma of Leydig and Sertoli cells of the testes. No traces of Bi were found in the liver.

### *Liberation of Bi ions in the animals intracranially exposed to Bi shotgun pellet*

Neurons and glia cells, in particular astrocytes, were heavily loaded in a zone around the implanted shotgun pellet (Fig. 2), but Bi-caused AMG staining was found everywhere in the CNS with motorneurons and ependyma cells being the most loaded. In the neocortex, where the implant was located, the strong spherical staining mentioned above looked like a halo around the pellet, getting broader the longer the animals survived, however, and increases in staining and amount of stained neurons were also obvious in the contralateral neocortex (Fig. 2), suggesting an axonal transport of liberated Bi

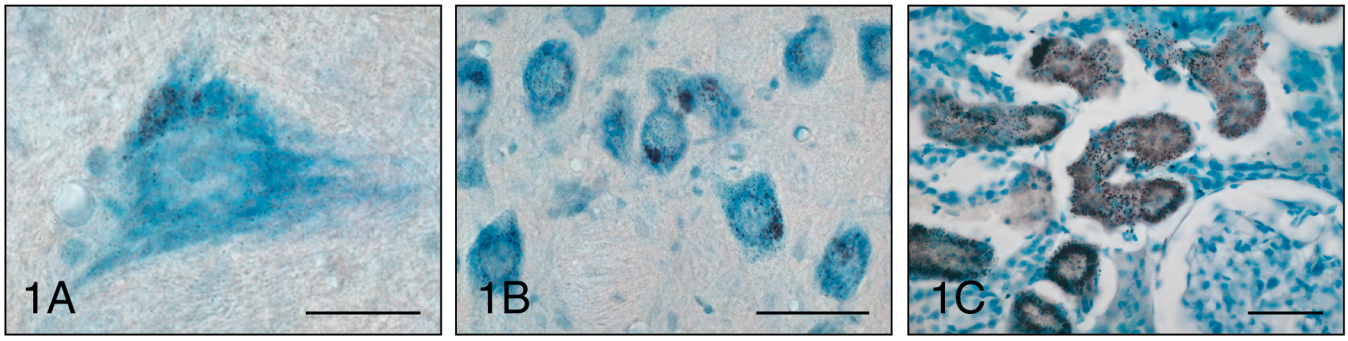


**Fig. 1.** Cryo-sections, 30 $\mu$m, from rats intramuscularly exposed to a single Bi shotgun pellet for 12 months. Tissue sections are AMG developed for 60 min and counterstained with toluidine blue. **A.** Black $Bi^{AMG}$ grains seen in a spinal cord motor neuron. Scale bar: 20 $\mu$m. **B.** Bismuth-loaded motor neurons in the facial nucleus. Scale bar: 50 $\mu$m. **C.** Tubular epithelial cells of the kidney heavily loaded with Bi. The glomerulus is void of AMG staining. Scale bar: 20 $\mu$m.

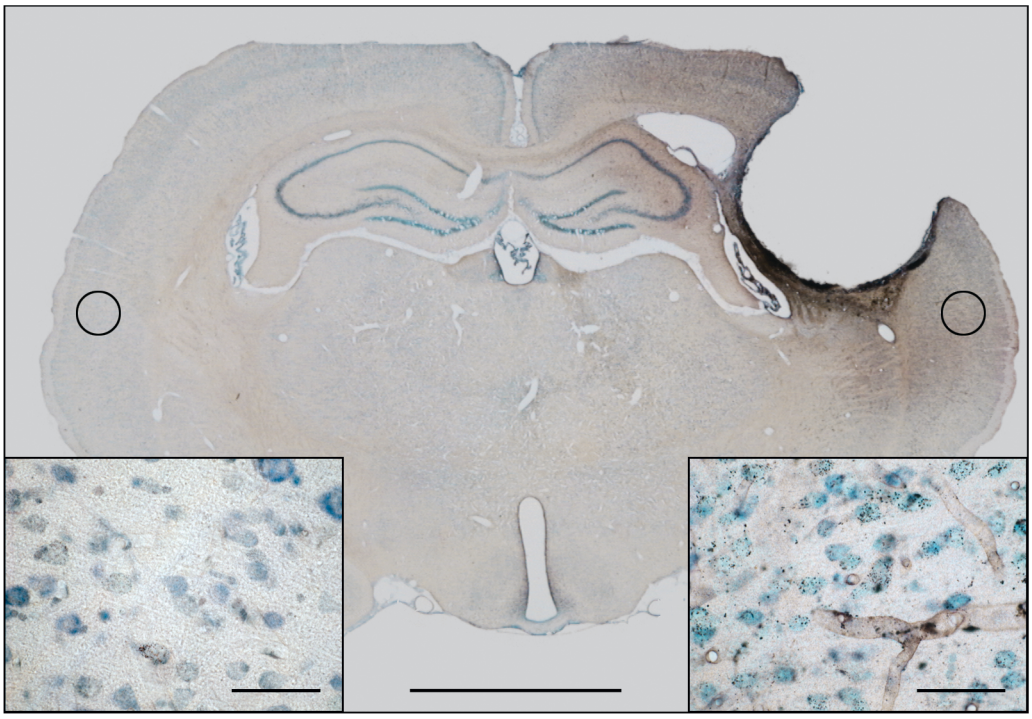

**Fig. 2. A.** Bi shotgun pellet was implanted in the neocortex and the rat was allowed to survive for 2 months. 30 $\mu$m frontal brain cryosections AMG developed for 60 min and counterstained with toluidine blue. Scale bar: 3 mm. Neurons and glia cells are heavily loaded in a spherical halo-like zone around the implanted shotgun pellet. Vessel walls ipsilateral to the implanted shotgun pellet also stain intensively (left insert, scale bar: 50 $\mu$m). Stained neurons can also be found contralaterally (right insert, scale bar: 50 $\mu$m) .

ions added to the blood-borne Bi ions. All animals demonstrated Bi uptake in the ependym lining the ventricular system, as did the choroid plexus (Fig. 3A).

The CA1 pyramidal cells of the hippocampus exhibited $Bi^{AMG}$ grains on both the ipsilateral and contralateral side in all the intracranially exposed animals (Fig. 3B) while the CA3 pyramidal cells were void of staining. Animals exposed to Bi for one month exhibited no AMG staining in the pyramidal cells or the deep nuclei of the cerebellum, whereas animals exposed for 2 and 2.5 months revealed $Bi^{AMG}$ grains in both areas (Fig. 3C).

The rats exposed to Bi for one month had neurons loaded with $Bi^{AMG}$ grains in the following brain stem nuclei: the red, the dorsal raphe, the oculomotoric, the abducens, the facial, the medial and lateral part of the vestibular, and the reticular magnocellular nuclei. However, 2 and 2.5months after implantation the amount and the intensity of the Bi load were even stronger, and the trochlear, trigeminal, and hypoglossal nuclei (Fig. 3D) were added to the group of stained nuclei. In the spinal cord the animals exposed to Bi for 2 and 2.5 months showed heavily loaded neurons (Fig. 3E), while the one-month exposed rats were void of staining.

In all animals Bi was detected in the tubular cells of the kidney (Fig. 3F), and staining intensity increased with time. No staining was found in liver or testes.

## Discussion

Histochemical tracing of Bi in the kidney after 6 months and after 12 months in the brain in rats with shotgun pellets implanted in their muscles demonstrated that Bi ions were liberated from metallic Bi and were taken up by specific cells of most organs. In other words, Bi is as little inert as metallic gold if placed in the living organism (Danscher, 2002). We do not know at the moment the exact mechanisms involved. We hypothesize that macrophages settle on the surface of the shotgun pellet and oxidize the metal by setting up small compartments with low pH and releasing anions, e.g. cyanide ions. The released Bi ions spread through the organism where certain cells take up Bi ions which most likely bind to SH-groups. Eventually, after having exerted its toxic effects on the molecular level, Bi ends up in lysosomes where the enzymatic breakdown of Bi-polluted proteins causes the formation of bismuth-sulphide clusters.

The toxic effect of Bi shotgun pellets has previously been investigated in mallards after intramuscular implantation or ingestion per os. The results made the authors conclude that Bi shotgun pellets did not elicit toxicity in mallard ducks or affect their reproduction or offspring (Sanderson et al., 1997, 1998).

In our laboratory, stereological analysis of spinal

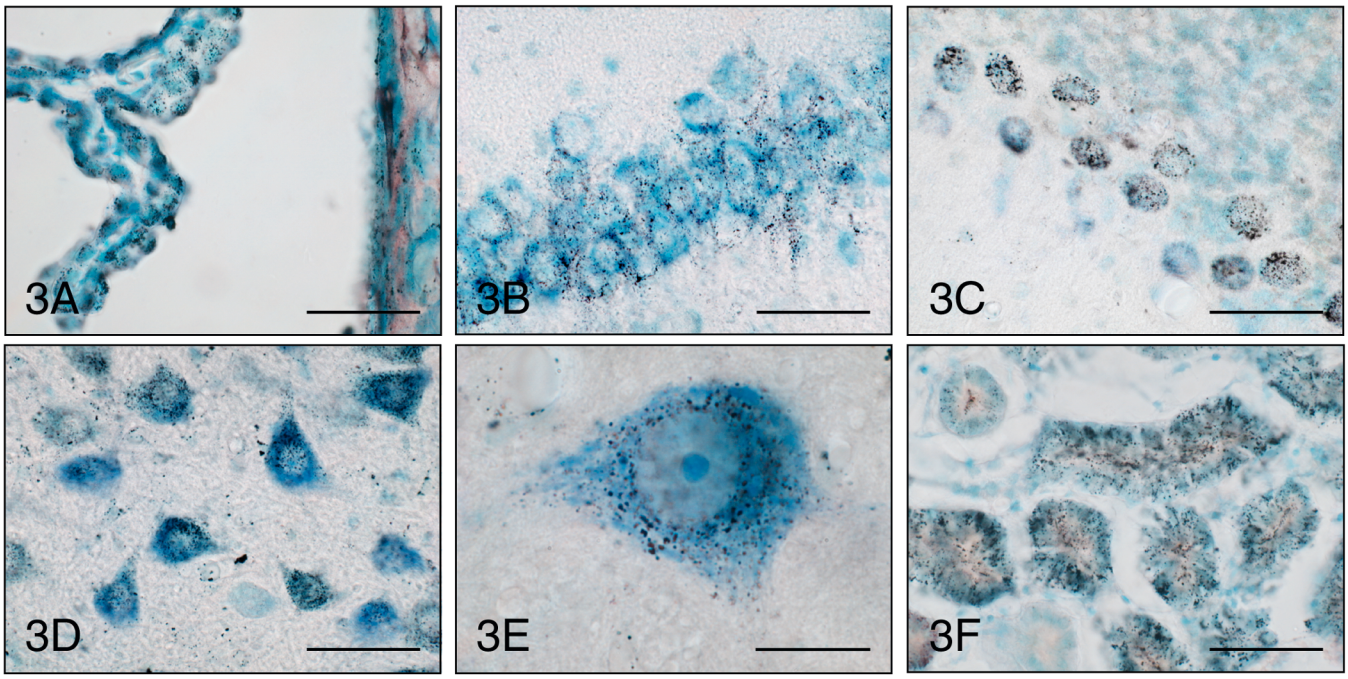


**Fig. 3.** The pictures are taken from rats allowed to survive for 2 months after implantation of a Bi shotgun pellet in the neocortex. 30-$\mu$m frontal brain cryosections, AMG developed for 60 min and counterstained with toluidine blue. **A.** Pronounced Bi uptake in the ependym lining the ventricular system and in the cubic epithelia covering the choroid plexus. Scale bar: 50 $\mu$m. **B.** Bismuth-loaded CA1 pyramidal cells of the hippocampus. Scale bar: 50 $\mu$m. **C.** Heavily stained pyramidal cells of the cerebellum. Scale bar: 50 $\mu$m. **D.** Massive accumulation of $Bi^{AMG}$ grains seen in motor neurons from the hypoglossal nucleus. Scale bar: 50 $\mu$m. **E.** High magnification of a spinal cord motor neuron containing numerous $Bi^{AMG}$ grains. Scale bar: 20 $\mu$m. **F.** Bi uptake in the tubular cells of the kidney (irrespective of survival time). Scale bar: 50 $\mu$m.

cord ganglia from rats intraperitoneally exposed to moderate levels of a Bi salt (bismuth subnitrate) revealed that approximately 20% of B-cells in the ganglia disappeared after 3 months, while A-cells seemed to be less harmed by Bi (Stoltenberg et al., 2003). These findings suggest that Bi-caused changes are subtle, and that quite serious damage can be overlooked, e.g. if histochemical and stereological tools are not implemented in the search. This is further supported by experiments where rats intraperitoneally exposed to bismuth subnitrate behaved, gained weight, moved, ate and drank like the control animals. AMG, however, revealed a selective uptake of Bi in the testosterone-producing Leydig cells with a parallel fall in blood testosterone (Stoltenberg et al., 2002). After 2 weeks the number of Leydig cells had been reduced by 17% indicating that Bi exposure causes the death of Leydig cells (Pedersen et al., 2003).

In conclusion, the finding that Bi ions released from Bi shotgun pellets implanted in animals are taken up by the brain and spinal cord as well as by other organs indicates that metallic Bi is at least potentially dangerous to the environment. Our findings suggest a need to further evaluate the behaviour and reproductive capability of animals exposed to Bi by drugs or implanted Bi shotgun pellets.

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